

AD _____

Award Number: W81XWH-04-1-0571

TITLE: Detecting and Targeting Oncogenic Myc in Breast Cancer

PRINCIPAL INVESTIGATOR: Linda Z. Penn, Ph.D.

CONTRACTING ORGANIZATION: University Health Network
Toronto, Ontario M5G 2C4
Canada

REPORT DATE: June 2005

TYPE OF REPORT: Annual

20060223 124

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 01-06-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 10 May 2004 – 9 May 2005	
4. TITLE AND SUBTITLE Detecting and Targeting Oncogenic Myc in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0571	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Linda Z. Penn, Ph.D. E-mail: lpenn@uhnres.utoronto.ca				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University Health Network Toronto, Ontario M5G 2C4 Canada				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT No abstract provided.					
15. SUBJECT TERMS Myc oncogene, transcription factor, chromatin immunoprecipitation, microarray, node-negative breast tumor, inhibitor screen, gene expression					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 15	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
Appendices.....	9

Introduction

Background: Deregulation of the cellular myc proto-oncogene is one of the strongest activators of tumorigenesis and understanding the target genes and pathways regulated by this transcription factor in cancer etiology will clearly mark a key advance. Myc expression and activity are highly restricted in normal human mammary epithelial cells (HMECs), but unleashed and deregulated in cells of malignant transformations. Because the protein product is identical in normal and tumor cells, three major issues arise. First, a definitive approach to detect oncogenic Myc in primary tumor specimens is severely lacking and long overdue. This issue has plagued the field during the two decades since Myc was first discovered. Second, it remains unclear whether Myc *function* is different in normal and tumor cells. Myc may regulate the same subset of target genes in both settings, but in a more robust manner in tumor cells. By contrast, deregulated, overexpressed Myc protein may bind and regulate an additional unique set of target genes in tumor cells. This issue has not yet been explored. Third, although it is clear that inhibiting Myc can trigger tumor regression and eradication in animal models, few initiatives are underway to target Myc as a therapeutic approach for human disease. Because Myc protein in normal and tumor cells is indistinguishable, it is thought that anti-Myc inhibitors would have little to no tumor specificity or therapeutic index. Clearly a novel approach is required.

Hypothesis: Our 'idea' is that in addition to the target genes regulated by Myc in non-transformed cells, constitutively activated and overexpressed Myc protein in tumor cells will directly bind and regulate a unique set of target genes that directly contribute to the carcinogenic process. For example, at high levels of expression, Myc may bind low affinity sites and regulate a distinct cohort of targets by a unique mechanism of action. By identifying this transformation specific subset of Myc target genes we aim to develop a diagnostic tool to identify oncogenic Myc *activity* in breast tumor cells. We also aim to develop a unique anti-cancer therapeutic that will potentially target this unique transforming activity of Myc. The TRRAP cofactor has been shown to be essential for Myc to drive transformation. This suggests blocking Myc:TRRAP interaction will inhibit the carcinogenic program directed by oncogenic Myc. By conducting the experiments outlined in this proposal we will test a unique hypothesis and will make significant contributions to the molecular diagnosis and treatment of breast cancer that can be applied to the clinic in a timely manner.

Specific Aims: Identify tumor-specific, directly-regulated Myc target genes in transformed HMECs and develop a definitive diagnostic tool to detect oncogenic Myc activity in breast cancer. Isolate small molecular weight inhibitors that can disrupt Myc:TRRAP interaction in vivo and identify Myc:TRRAP co-bound target genes in breast cancer.

Body

With support from the DOD, the research outlined in the original proposal has progressed in a steady and productive manner as expected for this first year of funding. To delineate the accomplishments to date, the tasks outlined in the original Statement of Work of the proposal are itemized below (*italics*) and a progress report for each task provided.

Statement of Work

Task 1: Identify tumor-specific, directly-regulated Myc target genes in transformed HMECs (months 1-36)

Directly compare fresh and formalin-fixed tissue for efficacy of Chip-on-chip assay using samples, including archived samples, prepared by the Andrulis group (months 1-6)

Complete: We anticipated that archived formalin-fixed tissue could be used for ChIP-on-chip analysis, however, over the course of the year, we have learned that formalin fixation conducted in pathology labs is not similar to the formaldehyde fixation used in our research lab as an early step of the ChIP-on-chip procedure. We suspect the issue has more to do with the heterogeneity in the methodology used by different technicians/residents over the years in various pathology labs (formalin formulation, time of fixation, etc). To overcome this problem, we evaluated whether frozen tissue could be used as an alternate source. We are pleased to report that we can achieve robust ChIP-on-chip results using 0.03g of frozen tissue (Fig 1). We will further determine the smallest amount of tissue required for this analysis. Importantly, we can move forward with the study, using frozen instead of formalin-fixed primary tissue for our ChIP-on-chip analysis.

Evaluate minimum numbers of cells that is required for quality assured Chip-on-chip of tissue sections, test reproducibility (months 6-12)

Complete: We have compared and modified two amplification methods to determine how few cells are required to achieve efficient ChIP-on-chip results without introducing bias due to amplification. Our results show that our 'random-priming' method shows outstanding reproducibility and minimal skewing due to amplification compared with the 'ligation-mediated PCR' approach, using our CpG island microarrays. Moreover, 10-7 or more cells provides robust results on a routine basis with a wide-variety of antibodies we are using in ChIP-on-chip.

Conduct the Myc specific Chip-on-chip assay in sixplicate with reverse dye labeling on one sample that has been processed for lasar capture microdissection for normal and tumor isogenic matched sample, in duplicate (months 12 -18) Yet to do.

Conduct Myc specific Chip-on-chip assay on 2 additional genetically similar patient samples (months 12-18) Yet to do

Conduct Myc specific Chip-on-chip on 3 similar samples whose underlying genetic abnormalities are distinct from the first series of 3 (months 18-24) Yet to do

Conduct data analysis to evaluate how target genes compare between isogenic normal and tumor matched material and between groups that harbor distinct genetic abnormalities and between all samples to identify a cohort commonly bound and regulated by Myc in tumor but not normal tissue. (months 24 -32) Yet to do

Further evaluate the diagnostic potential of this common cohort and evaluate whether cDNA expression profiling shows these targets are similarly regulated in ANN tumors of a specific subtype or genetic background (months 24 -32) Yet to do

Extend analysis to tissue arrays using in situ hybridization or IHC (months 24-36) Yet to do

Evaluate cDNA expression array data for genes identified as regulated by Myc in the MCF10A system (months 12-36) Yet to do

Task 2: Establish MCF10A cell system and identify tumor-specific, directly-regulated Myc target genes (months 1-36)

Introduce ectopic Myc expression in the MCF10A cells and evaluate biological effect at the level of proliferation, apoptosis induction in standard culture conditions (months 1-6)

Complete. We have ectopically expressed Myc in the MCF10A cells and have characterized cell growth and death, as proposed. Myc potentiates both cell proliferation and apoptosis, as expected. Results for apoptosis are shown (Fig. 2)

Conduct Myc ChIP-on-chip analysis and cDNA array analysis to identify target genes directly bound and regulated by Myc under asynchronous conditions (months 1-12)

Complete. We conducted ChIP analysis and identified H19 as a novel Myc target gene regulated by Myc in MCF10As. We further showed that Myc induction of H19 plays a role in Myc induced transformation by conducting siRNA knock-downs in breast cancer cell lines that do and do not express H19. Indeed, the loss of H19 inhibits full transformation potential of these breast cancer cells. Thus we show that we have identified at least one novel Myc-induced gene (H19), using the MCF10A system, that plays an important role in transformation (Fig. 3). We will further characterize additional Myc targets using the MCF10A system, identified using ChIP-on-chip.

Intro the control and Myc expressing cells introduce activatable erbB2 and p53-DD (months 6-12).

Nearly Complete. We have recently ectopically expressed the dominant negative p53-DD into these cells, as proposed and are in the process of introducing erbB2 using retroviral gene transfer technology. This will be complete in 1 to 2 months.

Assay cells for growth in soft agar as well as proliferation and apoptosis assays (months 12-18) Yet to do.

Assay Myc ChIP-on-chip and cDNA arrays on these cells expressing ectopic Myc and/or erbB2 and/or p53-DD, under asynchronously growing conditions (months 18-32) Yet to do.

Assay more transformed cells for invasion, polarity, morphology properties (months 18 - 32) Yet to do.

Test for genetic abnormalities and their effects on cell differentiation in matrigel (months 24-36) Yet to do.

Assay all cells for Myc Chip-on-chip and cDNA arrays when grown in soft agar (months 24-36) Yet to do.

Task 3: Isolate small molecular weight inhibitors that can disrupt Myc:TRRAP interaction in vivo and identify Myc:TRRAP co-bound target genes in breast cancer.

Develop and test antibodies to TRRAP for ChIP (months 1-12)

In progress. We raised antibodies to TRRAP and purchased commercially available antibodies to TRRAP. We compared these in ChIP reactions and our homemade antibody looked promising. We purified the rabbit antisera and further evaluated using a new, much more stringent quantitative-PCR based ChIP assay recently established in the lab. Unfortunately the sensitivity of the assay is not as robust as we had anticipated. To overcome this problem, we are evaluating a new cross-linking agent that has been reported to work well for molecules like TRRAP which do not contact DNA directly and therefore may need to be fixed to DNA through its protein:protein interactions using this new fixative. This is presently underway.

Develop Myc and TRRAP interacting fragments in new screening system and evaluate interaction and susceptibility to inhibition with TRRAP polypeptide (months 1-12)

In progress. Before setting up the screening system we needed to ensure the fragment of TRRAP shown to interact with Myc did indeed interact with Myc in vivo. Fragment II was shown in the yeast two hybrid repressed transactivator assay (RTA) to interact with Myc in vivo (Fig 4). The region of interaction was refined and fragment F8 was shown to interact with Myc in vitro (data not shown). We ectopically expressed F8 and show that interaction with exogenous Myc is evident by co-immunoprecipitation (Fig 5) and immunofluorescence (Fig 6). With these results we can now establish the screening system, as proposed.

Conduct screen and test positives in the RTA against Myc:TRRAP as well as other interactors of Myc and other interactors of TRRAP to evaluate specificity of the inhibitor (months 12-18) Yet to do.

Advance inhibitors to mammalian cell assays, including growth, transformation DNA binding using a Chip-on-chip approach (months 18-36) Yet to do.

Key Research Accomplishments

- We have learned that formalin-fixed tissues are not a useful source of material for ChIP analysis and have developed and characterized new methodology that allows ChIP analysis from frozen primary tissue.
- The MCF10A cells with and without ectopic deregulated Myc have been established and characterized. Moreover, we have identified H19 as a Myc-induced gene that plays an important functional role in the transformation of breast cells.
- Myc and TRRAP interaction occurs in vivo through MB2 and F8 regions, respectively, as evidenced by co-immunoprecipitation and immunofluorescence.

Reportable Outcomes

- Poster presented at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, June 8 - 11, 2005, Pennsylvania Convention Centre, Philadelphia, Pennsylvania

Conclusions

The work to date shows the expected progress and suggests the ultimate goals of the proposal will be accomplished during the tenure of this award. In the first year of funding specific objectives #1 and #2 have been initiated.

Identify tumor-specific, directly-regulated Myc target genes in transformed HMECs and develop a definitive diagnostic tool to detect oncogenic Myc activity in breast cancer.

Isolate small molecular weight inhibitors that can disrupt Myc:TRRAP interaction in vivo and identify Myc:TRRAP co-bound target genes in breast cancer.

To achieve our first specific aim, we have established the cell systems and technological tools and have identified at least one Myc target gene that contributes towards breast cancer development. We will build on this strong foundation to further identify the cohort of target genes bound and regulated by Myc to drive breast carcinogenesis.

As outlined in the second specific aim, we have further characterized the regions of Myc:TRRAP interaction and shown that they are functional in vivo in mammalian cells. With this knowledge we are now poised to establish and conduct a screen to identify small molecular weight inhibitors to disrupt Myc:TRRAP interaction in breast cancer cells.

Appendices

Figures 1 - 6

ChIP Tissue

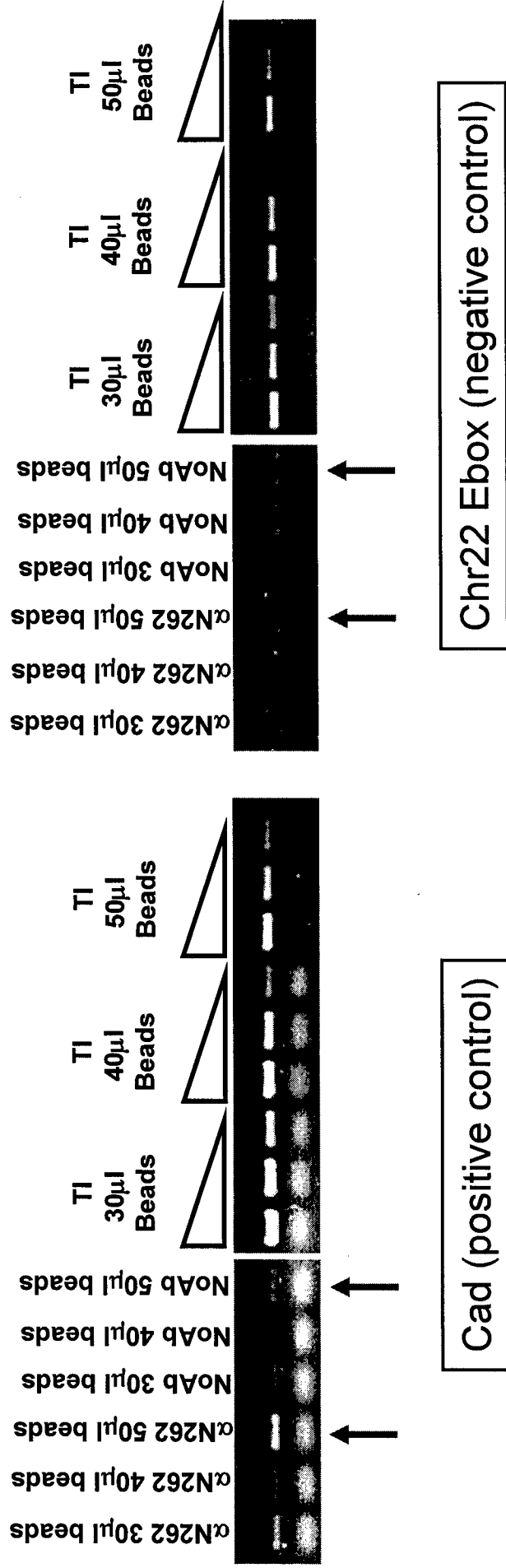


Fig 1A: Methodology to conduct Chromatin immunoprecipitation (ChIP) from frozen primary tissue has been established (see text for detail). Briefly anti-Myc antibody (N262) and no antibody control ChIPs from frozen primary tissue are evaluated for Myc-binding by PCR using primers to two potential genomic E-box binding sites: the cad gene (positive control) and chromosome 22 Ebox (negative control). In this experiment, the amount of protein G agarose beads are evaluated to optimize sensitivity and specificity of Myc-binding to the cad promoter from 0.03g of tissue. The red arrows indicate that 50 ul beads achieve these results. With this protocol established, we can proceed to conduct ChIP-chip in primary breast tissue (normal and tumor), as proposed.

Fig 1B: Chromatin immunoprecipitation Protocol For Frozen Primary Tissue

0.03g of tissue per antibody is chopped into small pieces in 1X PBS, formaldehyde is added to a final concentration of 1% at room temperature for 15 minutes. To stop the cross-linking reaction glycine is added to a final concentration of 0.125M for 5 minutes. Samples are washed one time in 1X PBS. The tissues are disaggregated in 1X PBS using a dounce homogenizer. The cells are collected by centrifugation and the pellet is resuspended in cell lysis buffer (5mM PIPES pH 8.0, 85mM KCL, 0.5% NP40, 0.5 mM phenylmethylsulfonyl fluoride, and 100 ng of leupeptin and aprotinin/ml), incubated on ice for 15 minutes. The nuclei pellets are collected by microcentrifugation and then resuspended in nuclei lysis buffer (50mM Tris-HCl pH 8.1, 10mM EDTA, 1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, and 100 ng of leupeptin and aprotinin/ml), incubated on ice for 20 minutes. Prior to sonication, 0.1g of glass beads (Sigma G-1277) are added to each sample. The samples are sonicated on ice at setting 10 for 7 pulses of 10 seconds to obtain an average of the sheared chromatin of approximately 600bp and 1000bp and then are microcentrifuged. The supernatant chromatin is incubated with 0.7µg of Myc N262 antibody (Santa Cruz) or no antibody and rotated at 4°C for approximately 12 to 16 h. 50 µl of Protein G-agarose beads are added to each sample, incubated on a rotating platform at 4°C for 3 hours. Immunoprecipitation, washing, and elution of immune complexes are carried out as described (Boyd, K. E. & Farnham, P. J. (1997). *Mol. Cell. Biol.* 17, 2529). Before the first wash, the supernatant from the reaction lacking primary antibody for each time point is saved as total input of chromatin and is processed with the eluted immunoprecipitates beginning at the crosslink reversal step. Crosslinks are reversed by addition of NaCl to a final concentration of 200 mM, and RNA is removed by addition of 10 µg of RNase A per sample followed by incubation at 65°C for 5 hr. Samples are then precipitated at -20°C overnight by the addition of 2 vol of ethanol and then pelleted by microcentrifugation. Samples are resuspended in 100 µl of TE (10 mM Tris, pH 7.5, 1 mM EDTA), 25 µl of 5X proteinase K buffer (1.25% SDS, 50 mM Tris, pH 7.5, 25 mM EDTA), and 1.5 µl of proteinase K (Boehringer Mannheim) and incubated at 42°C for 2 hr. Samples are extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and then precipitated with 1/10th vol of 3 M sodium acetate (pH 5.3), 5 µg of glycogen, and 2 vol of ethanol at -20°C overnight. Pellets are collected by microcentrifugation, resuspended in 30 µl of H₂O, and analyzed by using PCR. PCR reactions containing 2 µl of immunoprecipitate or diluted total input, 50 ng of each primer, 0.88 mM MgCl₂, 2 mM each dATP, dCTP, dGTP, dTTP, 1X Hot Star Taq Buffer and 1 unit of HOT STAR TAQ DNA polymerase (QIAGEN, 203205) in a total volume of 20 µl. After 32-35 cycles of amplification, PCR products are run on a 1% agarose gel and analyzed by ethidium bromide staining.

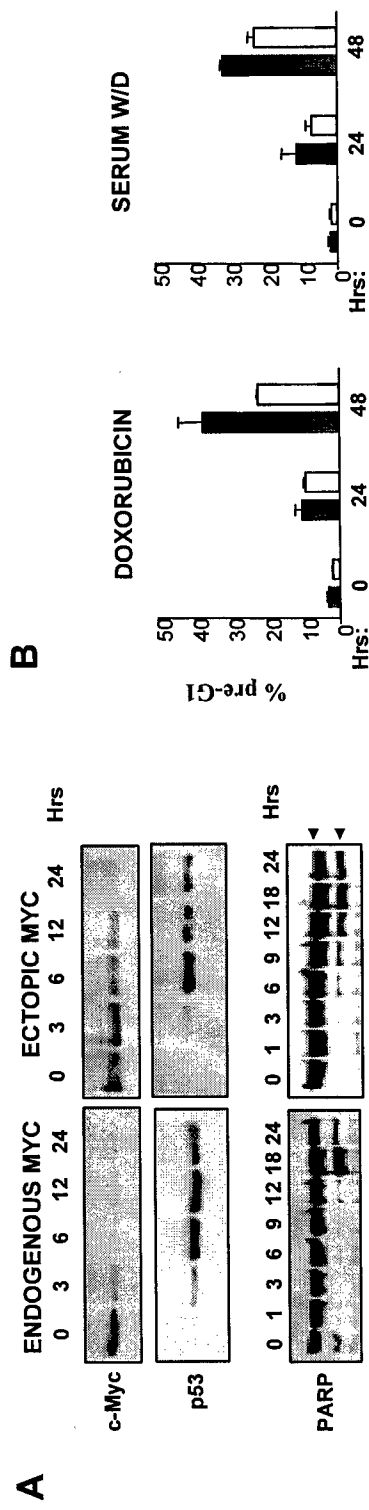


Figure 2. Ectopic Myc expression in MCF10A potentiates apoptosis A) Immunoblot analysis using antibodies specific for Myc, p53 and PARP, respectively, of protein isolated from control cells and cells expressing ectopic Myc that were exposed to doxorubicin for the indicated times. Panels on the left show control endogenous Myc expression whereas panels on the right demonstrate expression of ectopic Myc. B) Indicated cells with (black bars) and without (white bars) ectopic Myc expression were exposed to either doxorubicin or low serum for the indicated times. Cells were fixed and stained with propidium iodide and the percentage of cells with DNA content less than 2N is graphed as % pre-G1, indicative of the percentage of cells undergoing apoptosis in response to treatment.

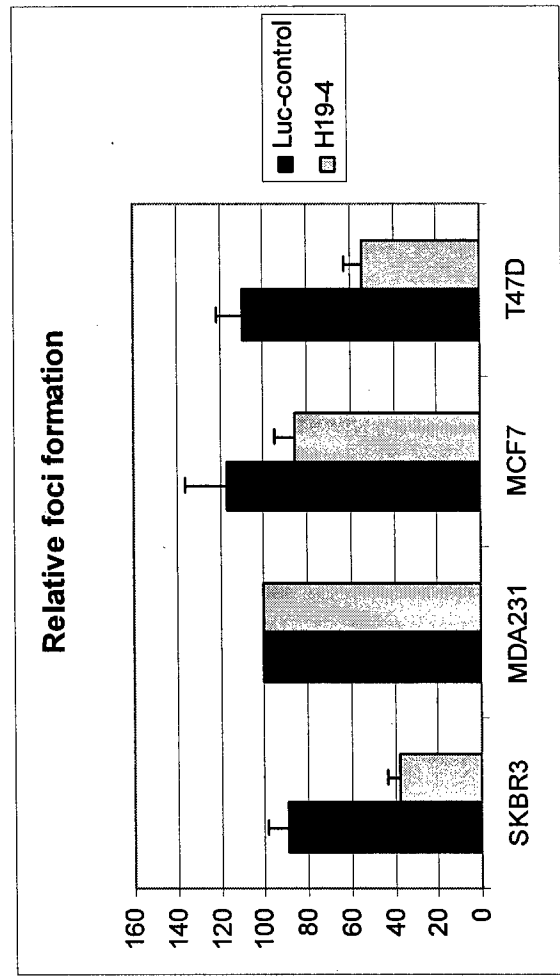
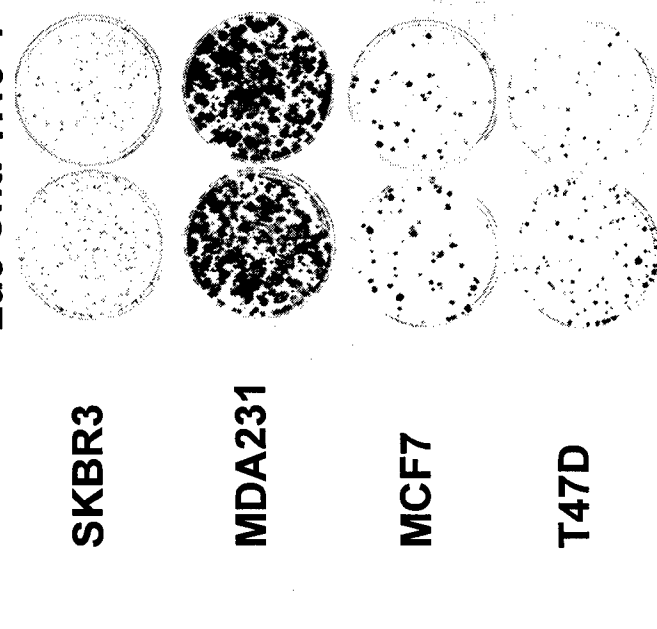
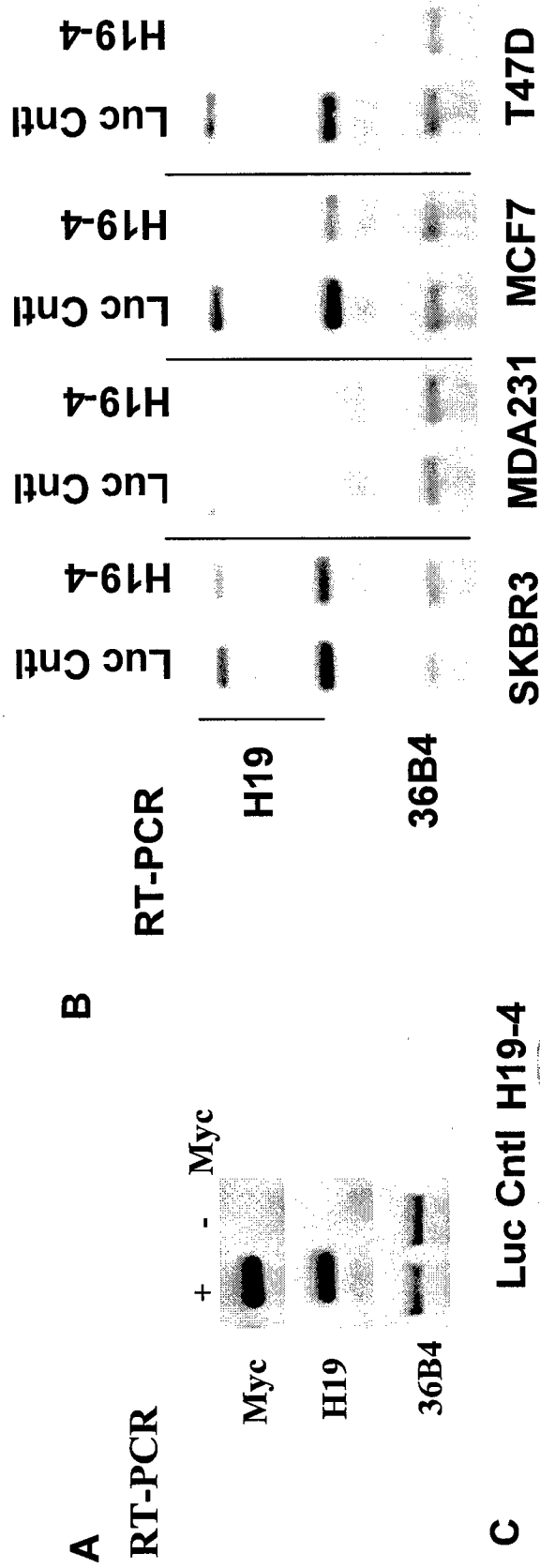
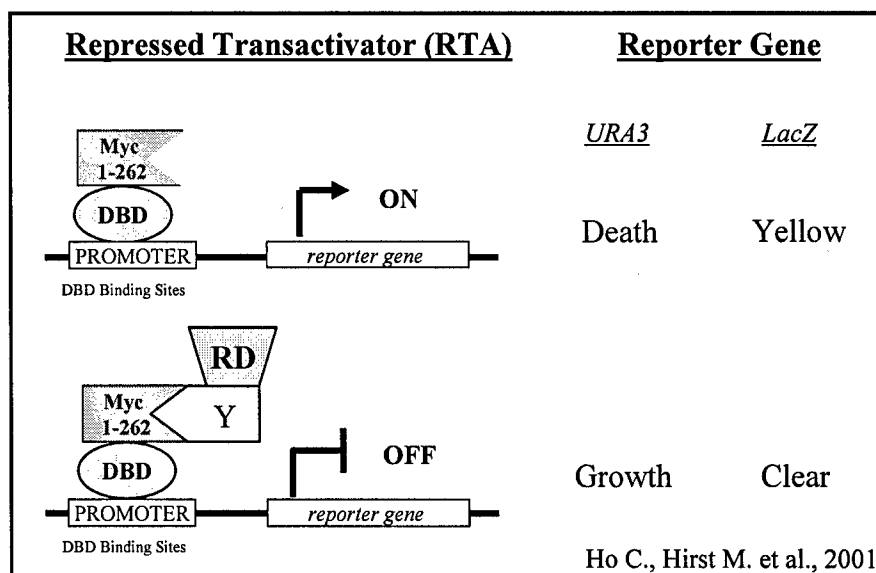


Fig 3: Myc induces H19 expression in MCF10As and knock-down of H19 decreases the transformation potential of breast cancer cell lines. A) Ectopic expression of Myc induces H19 expression in MCF10As, B) Knock down of H19 is achieved by siRNA to H19 (H19-4) but not control siRNA (Luc Cntl) in SKBR3, MCF7, T47D breast cell lines. C) Focus formation decreases in response to H19 knock-down. MDA231 cells do not express H19, and siRNA to H19 does not affect focus formation in these cells, serving as a specificity control for H19 siRNA.

A.

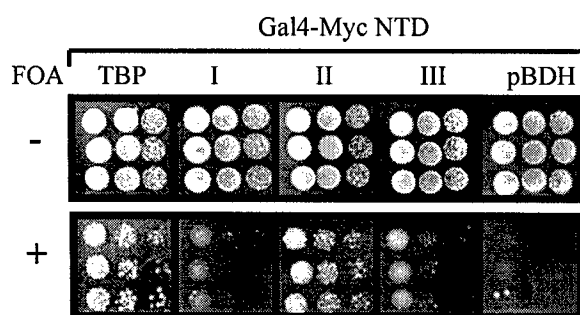


B.

TRRAP



C.



D.

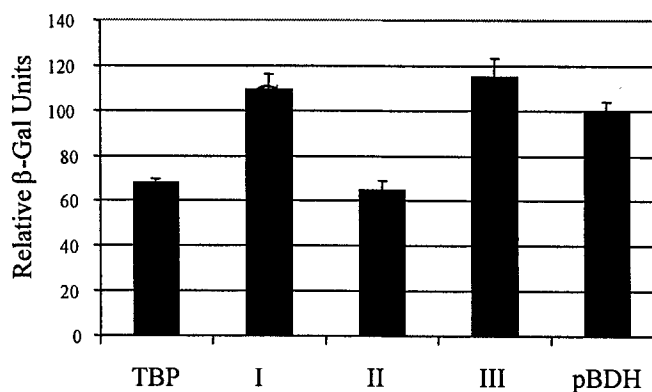


Figure 4: TRRAP fragment II interacts with Gal4-Myc NTD. (A) The RTA system was used to better define the region of TRRAP that specifically interacts with c-Myc N-terminal domain (Myc 1-262). Growth on FOA and inhibition of LacZ expression are indications of interaction between the bait and prey. (B) Schematic representation of TRRAP fragment I, fragment II and fragment III used in the RTA. Position of partial TRRAP fragments is outlined using black lines. (C) Gal4-Myc NTD bait was co-transformed into yeast cells with a TBP as a positive control, or TRRAP fragments I, II, III or a vector containing repression domain alone (pBDH). Resulting colonies were plated onto +/-FOA media for comparison of growth rescue. Only Yeast cells co-transformed with Fragment II and TBP together with MycNTD grew on FOA containing media. (D) Comparison of TBP and Fragment II suppression of *lacZ* expression by liquid β -galactosidase assay.

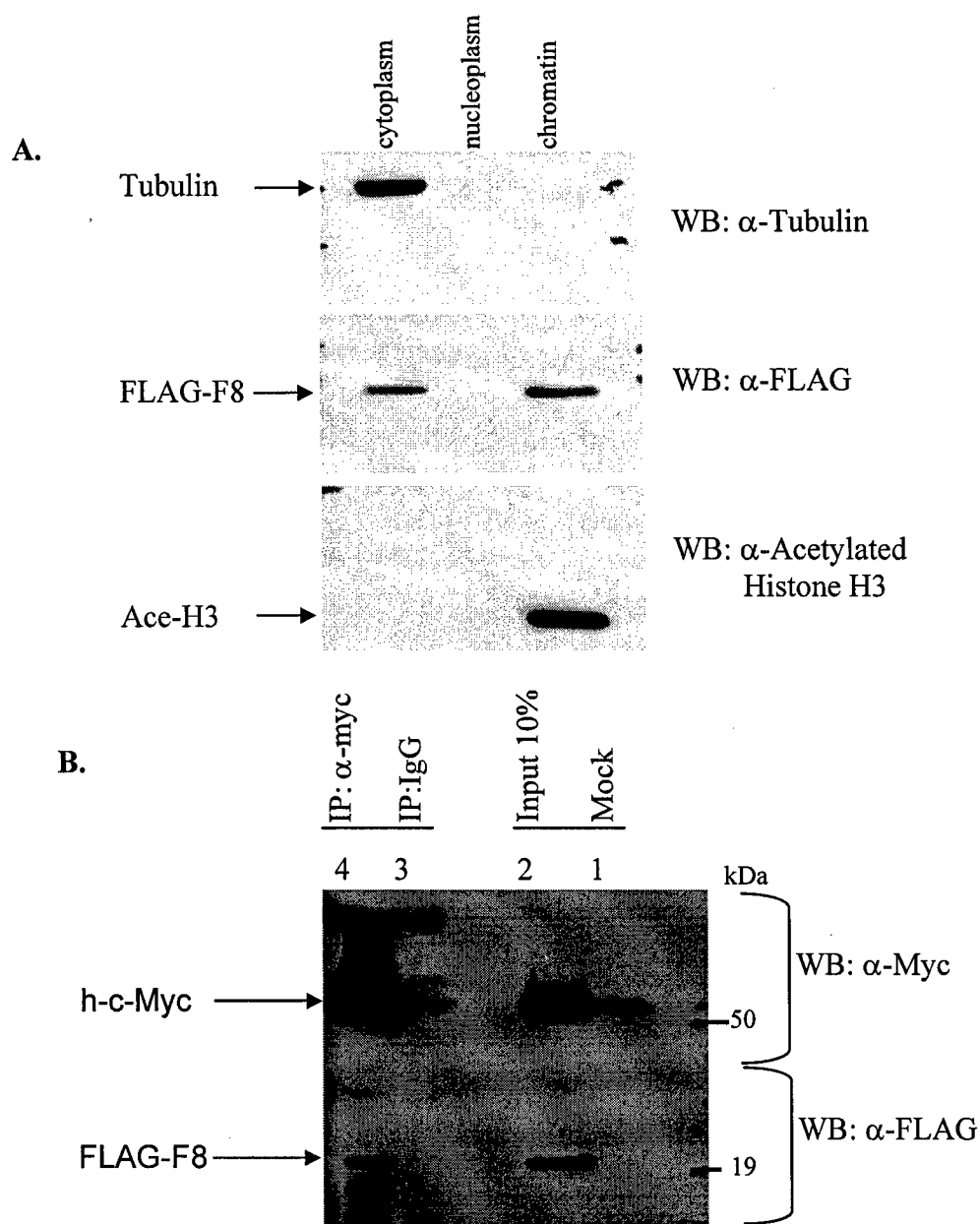


Figure 5. TRRAP fragment F8 is localized to the chromatin and interacts with c-Myc in mammalian cells. (A) 293TV cells transiently transfected with CMV10-F8 plasmid containing nuclear localization sequence (NLS) were harvested and sub-cellular fractions prepared. Equivalent protein amounts of cytoplasmic, nuclear and chromatin subfractions were separated on 12% SDS-PAGE gel. The blot was probed with anti-FLAG then stripped and subsequently re-probed with anti-Histone H3 and anti-alpha tubulin antibodies. (B) 293TV cells were transiently transfected with CMV10-F8 and pcDNA3-c-Myc. Total cell extract was prepared 48h after transfection and was subjected to immunoprecipitation with anti-c-Myc antibodies (N-262) or rabbit IgG. Co-immunoprecipitated F8 was visualized after SDS-PAGE and immunoblotting with anti-FLAG antibodies (bottom). The upper part of the blot was probed with anti-Myc antibodies (top); Input control (10%) is shown in the second lane while lysate of mock transfected cells is shown in the first lane. Migration of FLAG-F8 and c-Myc is indicated.

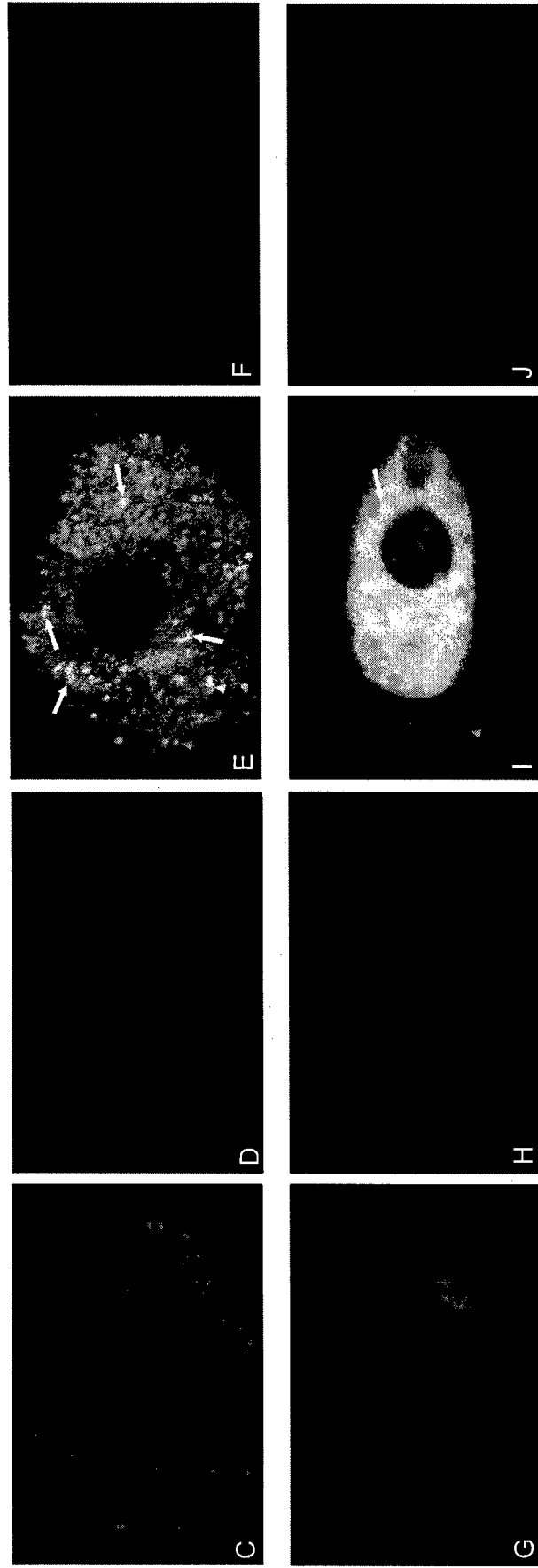


Fig. 6: F8 colocalizes with c-Myc in the nucleus. For immunofluorescence studies, 293TV cells (C, D, E, F) or HeLa cells (G, H, I, J) were transiently transfected with CMV10-F8 and pcDNA3-c-Myc for 24hs. Cells were fixed, stained with anti-FLAG monoclonal antibody (M2, Sigma) and rabbit polyclonal anti-Myc antibodies (N-262, Santa Cruz), and processed for confocal imaging. Green immunofluorescence staining shows nuclear localization of F8 and some cytoplasmic localization (C, G. See green arrowheads in E and I for cytoplasmic staining). Similar nuclear localization of c-Myc was shown by red fluorescence (D and H) as well as cytoplasmic localization in 293TV cells (red arrowhead in E). Merged fluorescence images indicate nuclear co-localization of F8 and c-Myc (white arrows in E and I) as well as some cytoplasmic co-localization in 293TV cells (white arrowhead in E). Dapi staining shows cells nuclei (F and J).